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A Sequential Native Chemical Ligation–Thiol-Michael Addition Strategy for Polymer-Polymer Ligation

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We systematically investigate sequential native chemical ligation (NCL)-thiol Michael addition as an efficient strategy for the dual modification of polymers. Polymers containing terminal cysteine functional groups were shown to undergo efficient NCL in the presence of amino acid-based and polymeric thioesters. The retention of the native cysteine side chain present at the NCL-junction distinguishes this approach from related multi-step strategies and was exploited for subsequent thiol-Michael additions providing access to diverse polymer architectures and polymer-peptide (single amino acid in this case) conjugates. Careful evaluation of model reactions involving a terminal cysteine functional poly(ethylene glycol), an amino acid thioester, i.e. phenylalanine thioester, and trifluoroethyl acrylate by NMR, SEC and MALDI-ToF MS revealed highly efficient modifications. Expansion of this concept towards oligomeric acrylates based on, e.g. N-acylated poly(amino ester)s (NPAEs) yielded Y-shape (co)polymers with quantitative conversions. The versatility and potential of the sequential modification was further demonstrated by employing a thioester-functionalised poly(2-ethyl-2-oxazoline) (PEtOx) to prepare a 3-arm mikto-arm star terpolymer.

Introduction

The desire to mimic nature's ability to create highly functional precision macromolecules has sparked the development of numerous synthetic tools in the last decades. In particular advancements in polymerisation techniques (e.g. in reversible deactivation radical polymerisations, RDRP) have opened up avenues to, e.g. precision and sequence-controlled macromolecules and single chain nanoparticles able to mimic the composition/sequence and folding of peptides and proteins, respectively.[1-4] Efficient coupling strategies have been extensively investigated to prepare even more sophisticated synthetic macromolecules with additional functionality or to synthesise biohybrids by combining synthetic and naturally occurring macromolecules such as peptides, proteins and carbohydrates.[5, 6] In recent years in particular, approaches which are (bio)orthogonal, selective/specific and do not require the use of a metal-catalyst have received significant attention.[7, 8] Amongst them, strain-promoted azide-alkyne cycloadditions and tetrazine-norbonene have been demonstrated to be useful tools which allow for (i) a straightforward and highly efficient modification of polymeric scaffolds, (ii) polymer-peptide/protein conjugations and (iii) polymer-polymer ligation.[9, 10]

In recent years, ligation strategies have been elaborated to include multi-step[11] and multi-component reactions[12] which can be employed to efficiently construct functionally complex and diverse macromolecules. The multi-step approach employs reactive molecules that contain latent functionality which can be accessed and exploited either during or post-polymerisation. A prominent example of this in polymer science is the use of homocysteine γ-thiolactone derivatives, which was introduced in 2011.[13] These reagents are cyclic thioesters that can undergo nucleophilic ring-opening resulting in the formation of a new carboxylic acid derivative and a free thiol. When the nucleophile employed is an amine, the reaction proceeds with formation of an amide bond and the thiol-containing side-chain formed is a methylene homologue of the side-chain of cysteine. The latent thiol functionality has been exploited for thiol-Michael additional,[14] radical thiol-ene[13]/yne[15] and disulfide bond formation.[16] The strategy has been employed for sequence-defined oligomer synthesis[17] and construction of dendrimers,[18] using iterative step-growth protocols, while thiolactone functional reagents for RDRP have also been synthesised providing access to functional polymer scaffolds.[19]

A much less regarded reaction in polymer science is native chemical ligation (NCL), which is frequently employed for the synthesis of peptides and proteins.[20, 21] Where the thiolactone group represents a protected thiol group and results in the formation of non-native, homologous cysteine derivatives, NCL enables the coupling of two completely unprotected peptide/peptidomimetic sequences. The only pre-requisite is that one sequence must contain an N-terminal cysteine residue, with the free amine and thiol groups necessary to promote the NCL mechanism, whilst the other sequence should contain a C-

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terminal thioester. The result of ligation is the formation of a native amide linkage, containing a native cysteine side-chain. Despite its efficiency, high-yielding character and mild conditions, only a small number of reports have described its application in polymer science,[22-25] with most of them being limited to the conjugation of peptides onto polymers.[26-28] The retention of the native thiol functionality of the cysteine, preserved at the ligation junction, predisposed for further functionalisation is an attractive feature of this strategy. Thus far, this has been exploited by peptide chemists to further expand the potential of the NCL,[29] e.g. through desulfurization,[30] methylation or Michael addition.[31]

However, in polymer science this functional handle has received close to no attention. To the best of our knowledge, only one report by Olsen and coworkers describes the general idea of exploiting the thiol group for further modifications.[32] The main focus of this report was on the synthesis of cysteine-functional RAFT polymers by RAFT and their site-specific expressed protein ligation, with efficiencies up to 60%. As part of this study one Y-shaped conjugate was prepared through dual modification of the cysteine functionality and assembled into nanoparticles. Thus, validating sequential NCL–thiol-ene modification as a viable strategy, analogous to the thiolactone strategy, for dual polymer modification of proteins.

Herein, we expand this concept and systematically investigate the sequential NCL–thiol-Michael addition approach for the efficient modification of polymers and demonstrate its modularity through the use of different polymeric building blocks. Specifically, cysteine-functional poly(ethylene glycol) (PEG-cysteine) is employed as the N-terminal cysteine containing sequence, whilst a model amino acid thioester and a thioester-functional poly(2-ethyl-2-oxazoline) are employed as the C-terminal sequence for the NCL reaction. The retained native cysteine side-chain is then functionalised by exploiting nucleophilic thiol-Michael addition reactions using a range of functional acrylates.[33] The potential of this strategy to prepare highly functional (co)polymers is exemplified by a sequential NCL–thiol-Michael addition strategy for the preparation of a functional miktoarm star-shaped terpolymer, which is achieved upon efficient coupling of macromolecular building blocks based on three functional polymer types, namely PEG-cysteine, a thioester modified poly(2-ethyl-2-oxazoline) (PEtOx-COSPh)[34, 35] and a N-acylated poly(amino ester) (NPAE) based acrylate (Scheme 1).[36]

Experimental

Materials

L-Cysteine, *N*-(*tert*-butoxycarbonyl)-L-phenylalanine, 1,1-carbonyldiimidazole (CDI), dimethylphenyl phosphine (DMPP), poly[ethylene glycol methyl ether acrylate] (PEGA M_n = 480 g/mol)) and 2,2,2-trifluoroethyl acrylate (TFEA) were obtained from Sigma-Aldrich and used as received. α-Methoxy-ω-amino-PEG (mPEG-NH_2) was obtained from RAPP Polymere and used as received. ω-carboxylic acid poly(2-ethyl-2-oxazoline) ($PEtOx_{30}$-COOH)[37] and N-acylated poly(aminoester) based oligomers (oligo(MeOx-alt-AA)$_n$A, oligo(EtOx-alt-AA)$_n$A, oligo(ButOx-alt-AA)$_n$A)[38, 39] were synthesised according to literature procedures.

Instruments and analysis

Nuclear Magnetic Resonance (NMR) spectroscopy was performed on Bruker AVANCE III HD-300 and HD-400 MHz spectrometers using deuterated solvents $CDCl_3$ and d_6-DSMO. Chemical shift values are given in ppm relative to residual solvent peaks. Size exclusion chromatography was performed on an Agilent Infinity II MDS instrument equipped with differential refractive index (DRI), viscometry (VS), dual angle light scatter (LS) and multiple wavelength UV detectors. The system was equipped with 2 x PL-gel Mixed C columns (300 x 7.5 mm) and a PL-gel 5 µm guard column. The eluent was THF with 2 % TEA (triethylamine) and 0.01 % BHT (butylated hydroxytoluene) additives. Samples were run at 1ml/min at 30 °C. Poly(methyl methacrylate) and polystyrene standards (Agilent EasyVials) were used for calibration (500 – 1,560,000 g/mol). Analyte samples were filtered through a GVHP membrane with 0.22 µm pore size before injection. Respectively, experimental molar mass ($M_{n,SEC}$) and dispersity ($Đ_m$) values of synthesised polymers were determined by conventional calibration using Agilent GPC/SEC software. Alternatively, an Agilent Infinity II MDS instrument equipped with differential refractive index (DRI), viscometry (VS), dual angle light scatter (LS) and variable wavelength UV detectors was employed. The system was equipped with 2 x PLgel Mixed D columns (300 x 7.5 mm) and a PLgel 5 µm guard column, with DMF containing 5 mmol NH_4BF_4 additive used as eluent. Samples were run at 1ml/min at 50 °C. Poly(methyl methacrylate) standards (Agilent EasyVials) were used for calibration between 500 - 955,000 g/mol. Analyte samples were filtered through a nylon membrane with 0.22 µm pore size before injection. Respectively, experimental molar mass ($M_{n,SEC}$) and dispersity ($Đ_m$) values of synthesised polymers were determined by conventional calibration and universal calibration using Agilent GPC/SEC software. Fourier-Transformed Infrared (FT-IR) absorption spectroscopy was performed on a Bruker VECTOR-22 FTIR spectrometer using a Golden Gate diamond attenuated reflection cell. MALDI-ToF spectra were recorded in reflection mode on a Bruker Daltonics Autoflex II MALDI-ToF mass spectrometer, equipped with a nitrogen laser delivering 2 ns pulses at 337 nm with positive ion ToF detection performed using an accelerating voltage of 25 kV. The matrix solution was prepared as a saturated solution in MeOH (Super-DHB) or THF (CHCA), at 40 mg/ml. The polymer samples were prepared at 10 mg/ml in either MeOH or THF, both the polymer and matrix solutions contained 5 mg/ml of Sodium Iodide. The polymer and matrix solutions were then mixed 50:50, and 0.5 microlitres of the resulting solution was spotted on an MTP 384 ground steel target plate. Calibration was performed with a poly(ethylene glycol) standards (M_n = 2000 and 4000 g/mol).

Experimental methods

Synthesis of 2,2-Dimethylthiazolidine-4-carboxylic acid (Tz4CA)[40]

Cysteine (10.0 g, 82.5 mmol) was added to acetone (1 L, 13.6 mol) and heated under reflux overnight. Upon cooling undissolved material was removed by filtration and the filtrate was concentrated by rotary evaporation to approx. 50 mL or just to the point of hot saturation. The solution was then cooled in a refrigerator over-night. Crystallisation occurred slowly in the form of long rectangular prisms, which tended to stick to the bottom of the flask. The crystals were filtered off, washed with a minimum of cold acetone, and dried in air. Recrystallisation in approx. 50mL of hot ethanol yielded Tz4CA (10.5 g, 64.3 mmol, 78 %) as white crystals. ^{1}H NMR (400 MHz, d_6-DMSO) δ_{ppm}: 1.44 (s, 3H, H_a), 1.60 (s, 3H, H_a), 2.97 (t, J_{HH} = 9.4 Hz, 1H, H_d), 3.35 (t, J_{HH} = 8.4 Hz, 1H, H_d), 4.00 (t, J_{HH} = 7.5 Hz, 1H, H_c); ^{13}C NMR (100 MHz, d_6-DMSO) δ_{ppm}: 30.4 (C_a), 32.7 (C_a), 39.7 (C_d), 64.9 (C_c), 76.3 (C_b), 173.1 (C_e).

Synthesis of 2,2-Dimethylthiazolidin-3-(N-formyl)-4-carboxylic acid (FTz4CA)[26]

Tz4CA (10.5 g, 64.3 mmol) was added to a solution of sodium formate (4.38 g, 64.3 mmol) in formic acid (83 mL, 2.20 mol) at 0 °C. Over a period of approximately 1 h, acetic anhydride (32 mL, 33.7 mmol) was added dropwise. The resulting mixture was stirred at room temperature for 1 h producing a white precipitate. Ice water (50 mL) was added and the precipitate was filtered and was washed further with ice cold water (10 mL). The dried powder was recrystallised in EtOH : H2O = 1 : 1 to yield FTz4CA (10.3 g, 55.4 mmol, 85 %) as colourless crystals. Spectroscopically FTz4CA was determined to be a 2.4 (maj) : 1 (min) mixture of cis/trans formamide isomers. ^{1}H NMR (400 MHz, d_6-DMSO) δ_{ppm}: 1.74 (s, 6H, $H_{a,\ min}$), 1.76 (s, 6H, $H_{a,\ maj}$), 3.12-3.48 (m, 2H, $H_{d,maj+min}$), 4.82 (dd, J_{HH} = 6.8, 2.7 Hz, 1H, $H_{c,maj}$), 5.07 (d, J_{HH} = 5.6 Hz, 1H, $H_{c,min}$), 8.23 (s, 1H, $H_{f,min}$), 8.41 (s, 1H, $H_{f,maj}$); ^{13}C NMR (100 MHz, d_6-DMSO) δ_{ppm}: 29.7 ($C_{a,min}$), 30.9 ($C_{d,maj}$), 31.0 ($C_{a,maj}$), 31.1 ($C_{d,min}$), 62.3 ($C_{c,maj}$), 65.5 ($C_{c,min}$), 70.1 ($C_{b,maj}$), 70.7 ($C_{b,min}$), 160.0 ($C_{f,maj}$), 161.0 ($C_{f,min}$), 171.1 ($C_{e,maj}$), 172.7 ($C_{e,min}$).

Synthesis of PEG-FTz4CA

Dry FTz4CA (1.14 g, 6.0 mmol, 3.0 eq) was dissolved in $CHCl_3$ (20 mL) under nitrogen. 1,1-Carbonyldiimidazole (0.97 g, 6.0 mmol, 3.0 eq) was added at RT and the solution was stirred for 90 min. mPEG-NH_2 (M_n = 2000 g/mol, 4.0 g, 2.0 mmol, 1.0 eq) was dissolved in $CHCl_3$ under nitrogen and added to the FTz4CA solution. The resulting mixture was stirred overnight at room temperature. The solution was then precipitated in cold Et_2O and isolated by Büchner filtration. The purification process was repeated three times. The isolated precipitate was then washed with ice cold Et_2O and dried in an oven at 70 °C to yield **1** as a white solid (3.5 g, 1.6 mmol, 81 %).

Synthesis of PEG-cysteine (2)

PEG-FTz4CA (3.0 g, 1.38 mmol) was dissolved in 0.5 M HCl (15 mL) and heated at 70 °C for 12 h. The solution was then dialysed against diluted acetic acid (0.01 M). The product **2** was isolated by lyophilisation.

Synthesis of phenyl 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanethioate (BocPheSPh)[41]

N-(*tert*-butoxycarbonyl)-L-phenylalanine (2.0 g, 7.5 mmol) was dissolved in dry THF (150 mL) and *N*-methylmorpholine (0.99 mL, 9.0 mmol) was added. The resulting solution was cooled to 0 °C prior to addition of isobutyl chloroformate (1.08 mL, 8.3 mmol) then stirred for 15 min. Thiophenol (0.92 mL, 9.0 mmol) was added followed by a second portion of *N*-methylmorpholine (0.99 mL, 9.0 mmol). The resulting solution was then allowed to reach room temperature and stirred overnight. The reaction mixture was filtered and concentrated to give a crude residue which was dissolved in DCM and washed with $NaHCO_3$, H_2O and NaCl. The organic phase was collected, dried over $MgSO_4$ and concentrated to yield the crude product which was purified by flash chromatography (EtOAc/hexane) to yield the pure thioester as a white solid (1.9 g, 5.3 mmol, 70%). ^{1}H NMR (300 MHz, $CDCl_3$) δ_{ppm}: 1.47 (9H, s, H_a), 3.17 (2H, m, H_c), 4.79 (1H, m, H_b), 5.01 (1H, br, H_N), 7.33 (10H, m, H_{Ar}); ^{13}C NMR (75 MHz, $CDCl_3$) δ_{ppm}: 28.3 (C_a), 38.4 (C_C), 61.0 (C_b), 80.5 (C_f), 127.2 (C_{Ar}), 127.4 ($C_{Ar,q}$), 128.7, 129.3, 129.4, 129.5, 134.6 (C_{Ar}), 135.6 ($C_{Ar,q}$), 155.0 (C_e), 199.4 (C_d).

General procedure for native chemical ligation

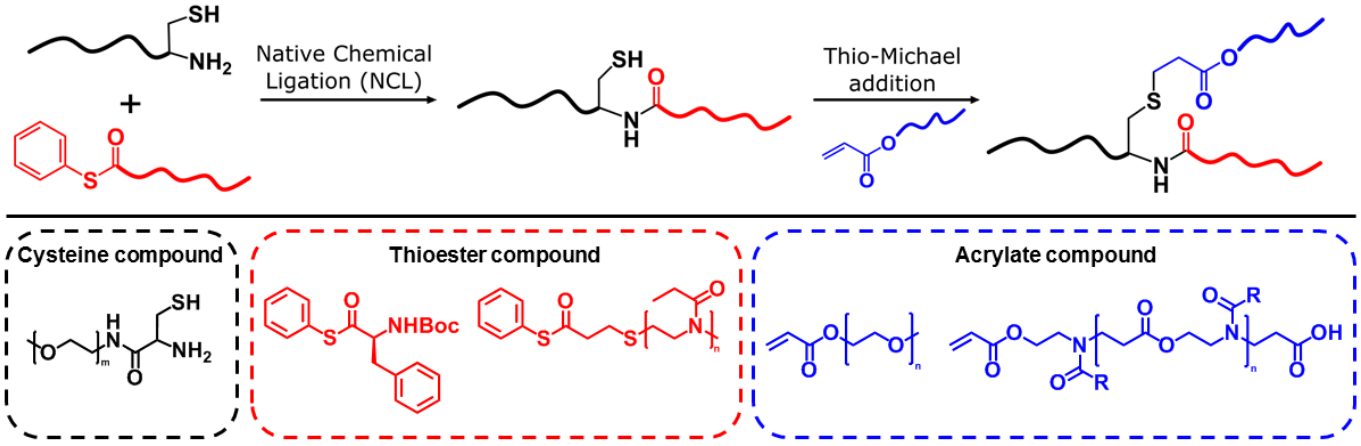
Polymer **2** (1.0 eq) was dissolved in MeOH (0.03 mmol/mL) and $NaBH_4$ (2.0 eq) was added slowly to reduce any disulfide (**2'**) and prevent any oxidation during the reaction. The resulting mixture was stirred at room temperature for 1 hr under N_2. Thioester (1.0 eq) was dissolved in minimal amount of deoxygenated (N_2 bubbling) MeOH and added to the reaction mixture which was then left allowed to stir at room temperature overnight. The crude reaction mixture was dialysed against H_2O and lyophilised to yield the pure product.

General procedure for thiol-Michael addition

Polymer **3** (1.0 eq) was dissolved in dry DMSO (0.01 mmol/mL) and deoxygenated by bubbling with N_2. An aliquot of a deoxygenated (N_2 bubbling) DMPP stock solution in DMSO (5.0 eq, DMPP) was added the resulting mixture was stirred for 90 min. Chosen acrylate (1.5 eq) was dissolved in a minimal amount of DMSO and deoxygenated by bubbling with N_2 before addition to the reaction mixture, which was allowed to stir at room temperature overnight. The crude reaction mixture was then dialysed against H_2O and lyophilised to yield the pure product.

Synthesis of $PEtOx_{30}$-COSPh (8)

$PEtOx_{30}COOH$ (200 mg, 64.7 µmol) was dissolved in dry THF (1.0 mL) and *N*-methylmorpholine (14 µL, 129.4 µmol) was added. The resulting solution was cooled to 0 °C prior to addition of isobutyl chloroformate (17 µL, 129.4 µmol) then stirred for 15 min. Thiophenol (13 µL, 129.4 µmol) was added followed by a second portion of *N*-methylmorpholine (14 µL, 129.4 µmol). The resulting solution was then allowed to reach room temperature



Scheme 1 Schematic representation of the Native Chemical Ligation–Thiol-Michael addition strategy (top) and the individual components used in this study (bottom). The cysteine component is shown as the protected version, namely thiazolodine, which is deprotected before the NCL.

and stirred overnight. The reaction mixture was filtered and concentrated to give a crude residue which was dissolved in DCM and washed with $NaHCO_3$, H_2O and NaCl. The organic.phase was collected, dried over $MgSO_4$ and concentrated to yield the crude product which was purified by precipitation in cold Et_2O to yield the pure thioester (8) as a white solid

Results and discussion

Initially, in order to synthesise PEG-cysteine, the nucleophilic thiol and amine groups of cysteine were protected as the formyl-thiazolodine cysteine derivative (FTz4CA) (Fig. S1 – S2).[26, 40] PEGylation of FTz4CA was achieved via amidation using α-methoxy-ω-amino-PEG (mPEG-NH_2, M_n = 2000 g/mol), followed by aqueous acid deprotection of the formylated thiazolodine end-group (Fig. 1). The successful formation of PEG-FTz4CA (**1**) was confirmed by appearance of the methoxy and ethylene glycol protons at 3.24 and 3.51 ppm respectively in the ^{1}H NMR (Fig. S3A).

Deprotection of the thiazolodine group to yield PEG-cysteine (**2**) proceeded with an up-field shift of the α-methine proton from 4.74 to 3.93 ppm, and disappearance of the formyl and acetyl protons at 8.35 and 1.76 ppm respectively (Fig. S4A). Size exclusion chromatography (SEC) revealed an unexpected increase in molecular weight upon deprotection with the $M_{n,SEC}$ of **1** increasing from 3700 g/mol to 6800 g/mol upon formation of **2** (Fig. 1A). The increase in M_n coincided with an increase in the molecular weight distribution (M_w/M_n; $Đ_m$) from 1.06 to 1.13 arising from the bimodal shape of the chromatogram.

Matrix-assisted laser desorption/ionisation time of flight mass spectroscopy (MALDI-ToF-MS) was used to further characterise the sequential chain-end modification of mPEG-NH_2 to **1** (Fig. 1B) and then **2** (Fig. 1C). Formation of **1** was confirmed by a shift in m/z from 1859.166 to 2030.301 (n = 41) (Table S1, Fig. S3C). The new distribution exhibited a repeating mass unit of 44 Da, corresponding to the ethylene glycol repeating unit of the PEG chain. Likewise, the deprotection of the formyl and thiazolidine groups was confirmed by the shift in m/z from 2030.301 to 1962.119 (n = 41) with retention of the 44 Da repeating unit of the PEG chain (Fig. 1C, Table S1, Fig. S4C). However, in agreement with the SEC data, the MALDI spectrum of **2** revealed a second distribution at approximately double the molecular weight (Fig. 1C, Fig. S4D). It was hypothesised that the increase in M_n and $Đ_m$, as well as the second distribution in the MALDI spectrum, was a result of disulfide formation due to oxidation occurring during purification (*vide infra*).

To investigate the potential of **2** to undergo NCL, Boc-protected phenylalanine (BocPhe) was converted to its thioester, phenyl 2-((tert-butoxycarbonyl)amino)-3-phenylpropanethioate (BocPheSPh), which was obtained with spectroscopic purity (Fig. S5).[41] Phenylalanine was selected as a model for larger peptides/protein-based thioesters accessible via peptide synthesis and protein engineering and expression respectively.[42, 43] Sodium borohydride ($NaBH_4$) was used to reduce **2'** to **2** *in situ* and ensure retention of both the free thiol and amine group, both of which are essential for NCL to occur. The aromatic group, present in the side chain of Phe, and the Boc-protecting group, provided a useful spectroscopic handle to help confirm success of the NCL reaction by ^{1}H NMR with appearance of the aromatic and methyl protons at 7.24 and 1.29 ppm respectively (Fig. S6A). Further evidence and quantification of the NCL reaction was provided by the change in chemical shift and relative integrals (1 : 1) of the α-protons of the Phe (4.79 to 4.54 ppm) and Cys (3.93 to 4.19 ppm) residues respectively.

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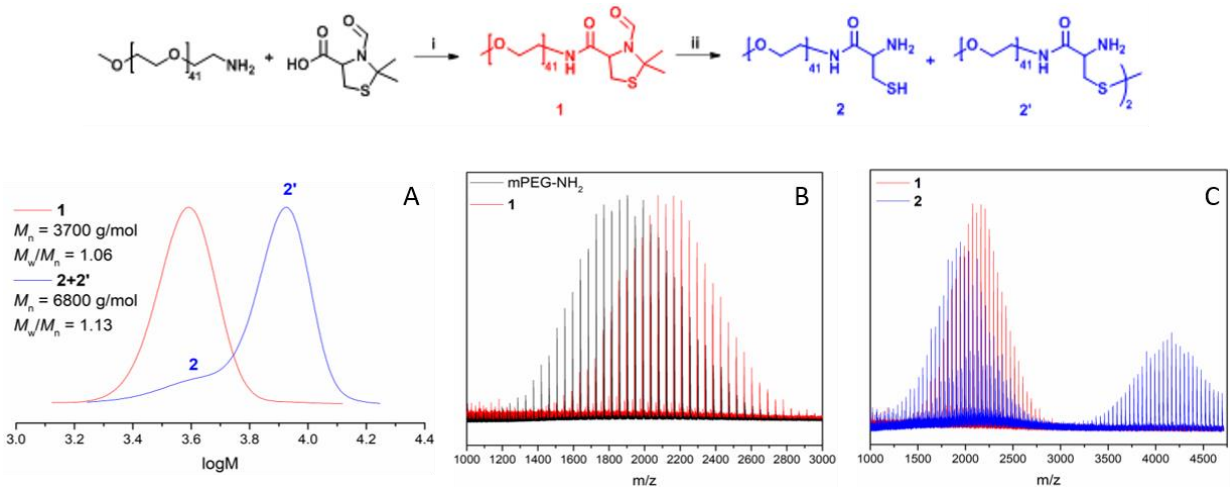

Fig. 1 Synthesis of terminal cysteine-functional PEG. Two step synthesis, i) CDI, $CHCl_3$, RT, ii) HCl (0.5 M), 70 °C; (A) SEC traces, DMF; (B, C) MALDI spectra of the protected (**1**) and deprotected (**2, 2'**) compound.

Molecular weight analysis by SEC furnished a bimodal distribution attributed to the formation of the desired NCL product in its reduced (**3**) and oxidised (**3'**) form (Fig. S7), whilst MALDI-ToF-MS demonstrated the expected increase in the m/z from 1962.119 to 2209.118 (*n* = 41, Table S1, Fig. S8).

An attractive feature of the NCL reaction is the retention of the free thiol group of the native cysteine side chain, which from a synthetic perspective, can be exploited for further chemistry. However, in this work it has been hypothesised that the majority of the free thiol group in **3** is consumed by oxidation to **3'** during purification. To test this hypothesis an SEC experiment was performed in which a batch of polymer **3/3'** was dissolved in THF and disulfide reducing agent dimethylphenylphosphine (DMPP) was added at a range of concentrations (0.5 – 5.0 eq).

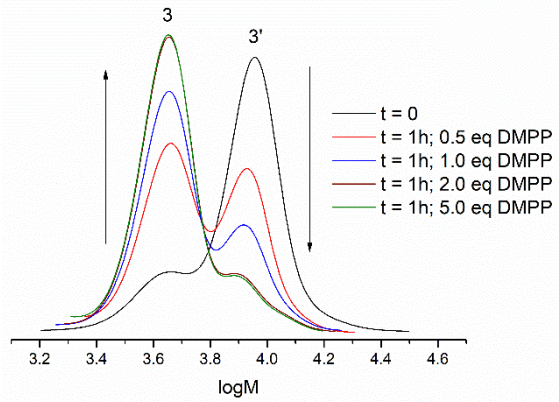

Fig. 2 SEC (THF) chromatograms at t = 1h, demonstrating the in-situ reduction of **3**' to **3** in the presence of increasing amounts of DMPP.

At (sub)stoichiometric amounts (0.5 – 1.0 eq), reduction of **3'** to **3** was complete within 48 hours with a corresponding molecular weight decrease from M_n = 7100 g/mol to M_n = 4400 g/mol (Fig. S9). Increasing the stoichiometric excess of the DMPP (2.0 – 5.0 eq) increased the rate of reduction such that complete reduction of **3'** to **3** was achieved within 1 hour (Fig. 2).

Phosphines can also catalyse nucleophilic thiol-Michael additions so reduction of **3'** using DMPP (0.15 – 5.0 eq) was screened in the presence of trifluoroethyl acrylate (TFEA) to investigate the potential for *in situ* reduction and thiol-Michael addition. The fluorine atoms present in TFEA provide a unique spectroscopic handle to help monitor the thiol-Michael addition reaction. Thus, the reaction between **3** and TFEA, in the presence of DMPP, was confirmed by the shift of the CF_3 signal from -72.65 to -72.32 ppm in the ^{19}F NMR spectrum (Fig. S10). This was supported by ^1H NMR with a shift of the vinyl protons of TFEA from 6.0 – 6.6 ppm to 2.5 – 3.0 ppm, and the appearance of a quartet corresponding to the α-methylene protons of TFEA at 4.74 ppm (H_q, Fig. S11). The efficiency of the thiol-Michael addition reaction was quantified by comparison of the integral of this signal with the signal corresponding to the aromatic protons of the Phe residue at 7.24 ppm (H_a, Fig S11). At substoichiometric concentrations of DMPP (0.15 – 0.75 eq), the efficiency of the thiol-Michael addition ranged from 12% to 78%, whilst when 1.5 eq and 5.0 eq of DMPP were employed, the efficiency increased to 86% and 91%, respectively (Fig. 3).

MALDI-ToF-MS further confirmed that the thiol-Michael addition between **3** and TFEA was the major product of the reaction. When an excess of the DMPP was employed (5.0 eq), the major distribution corresponded to the product of the thiol-Michael addition, **4** (Fig. 4). Zooming in to the region m/z = 2300

- 2400 allows adduct **4** corresponding to n = 41 (Table S1) to be identified (m/z_{th} = 2363.260; m/z_{obs} = 2363.406, Fig. 2C). The mono-modal distribution observed in the MALDI is replicated in the SEC (Fig. S12). Formation of **4** also proceeds with a change in molecular weight from M_n = 7100 g/mol (**3**+**3'**) to M_n = 6600 g/mol (**4**).

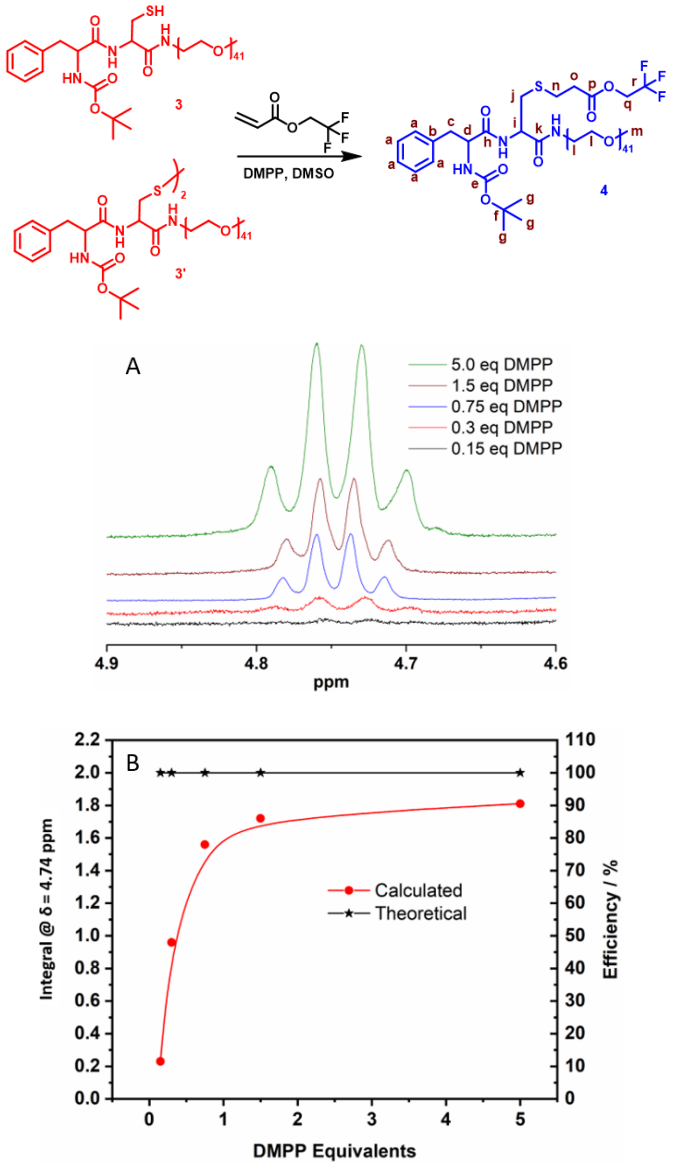

Fig. 3 Evaluation of the thiol-Michael addition modification of NCL product (**3**) with TFEA. (A) ^{1}H NMR at δ = 4.74 ppm, corresponding to H_q in the modified polymer **4**; (B) graphical representation of the efficiency of the thio-Michael reaction as a functional of the DMPP concentration.

The reaction conditions were then adopted for the thiol-Michael addition of **3** with polymeric acrylates (summarised in Table 1). From a bioconjugation point of view, this represents a potential approach to controlling the architecture (Y-shaped vs. linear) and composition of peptide/protein-polymer conjugates. Initially, acrylic acid terminated N-acylated poly(amino ester) (NPAE)-based macromonomers (oligo(MeOx-alt-AA)$_n$A, and oligo(EtOx-alt-AA)$_n$A), previously reported as heterotelechelic monomers for radical polymerisations, were investigated.[38, 44]

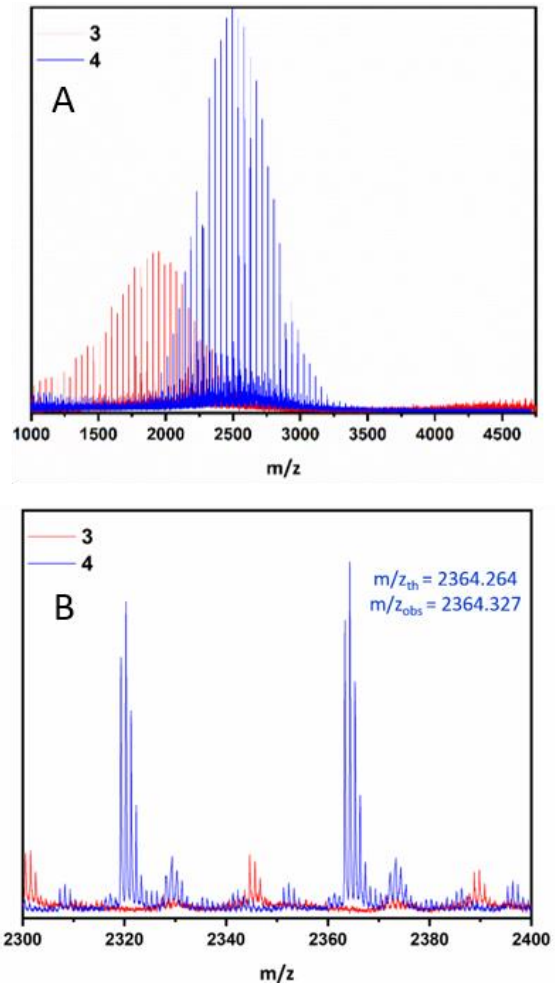

Fig. 4 MALDI-ToF-MS spectra showing the formation of **4** (blue) from **3** (red). (A) complete spectrum; (B) zoom into the region between m/z = 2300 – 2400 showing **4** (n = 40 - 41).

The progress of the thiol-Michael addition reaction was again monitored by the disappearance of the vinyl proton signals at 6.0 – 6.5 ppm. The final conversion was quantified by comparing the integral corresponding to the α-methine proton at 4.42 ppm of the Phe group, with the pendant methyl proton signals of the oligo(MeOx-alt-AA)$_n$A (**5**, 96 %) at 2.00 ppm (Fig. S13A), and oligo(EtOx-alt-AA)$_n$A (**6**, >99 %) at 0.96 ppm (Fig. S14A). The $M_{n,SEC}$ decreased upon formation of both **5** (4700 g/mol, Fig. S13B) and **6** (4800 g/mol, Fig. S14B), which is consistent with reduction of **3** (7100 g/mol) followed by thiol-Michael addition to oligo(MeOx-alt-AA)$_n$A (M_n = 384 g/mol) and oligo(EtOx-alt-AA)$_n$A (M_n = 414 g/mol) respectively.

The formation of **5** and **6** represents the formation of block copolymers (PEG-*b*-oligo(MeOx-alt-AA)$_n$A/oligo(EtOx-alt-AA)$_n$A) with the Phe residue at the junction between the two polymers. Alternatively, by using differently sized PEGA for the thiol-Michael addition, PEG homopolymer equivalents with the pendant Phe group (and potentially peptides), at variable positions along the polymer backbone are accessible. Exemplarily, the addition of PEGA (M_n = 480 g/mol) resulted in

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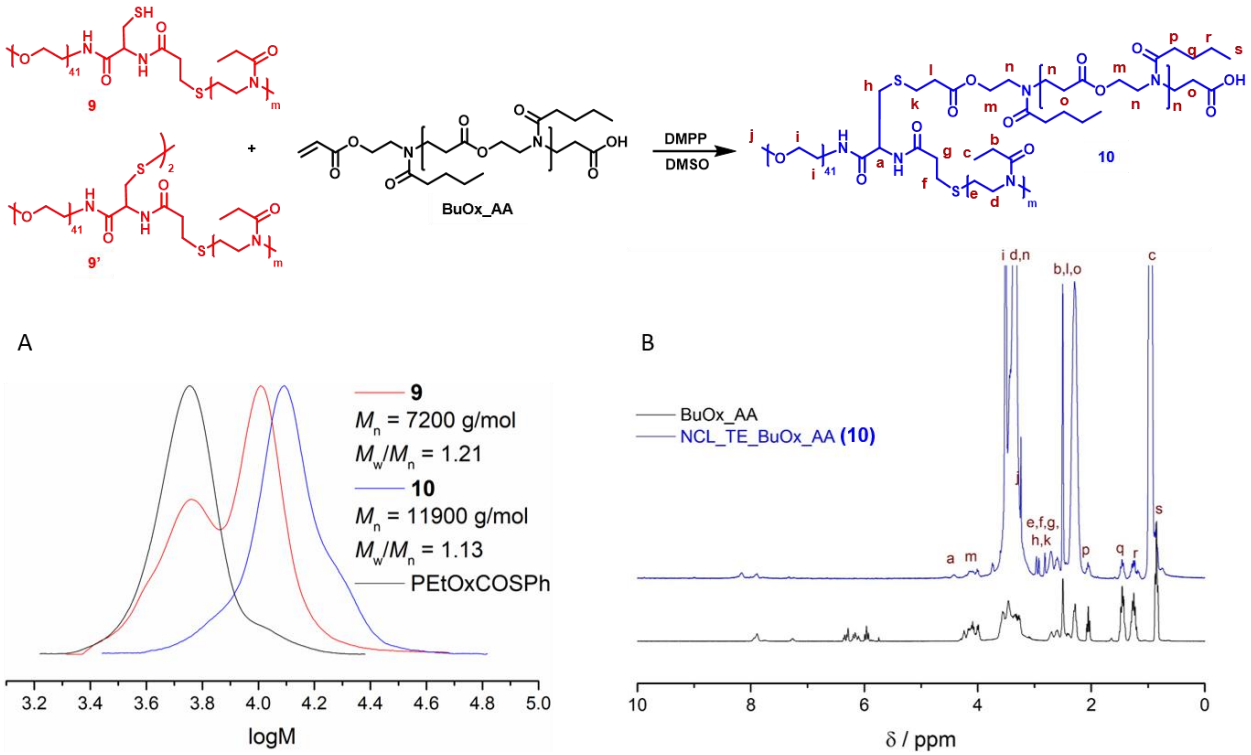

Fig. 5 Synthesis of 3-arm miktoarm star terpolymer μ-(PEG,PEtOx,NPAE) (**10**). Thiol-Michael addition of **9**,**9'** and oligo(ButOx-alt-AA)$_n$A); (A) SEC traces and (B) ^{1}H NMR spectra.

the formation of **7** with > 99% conversion according to the relative integrals of the α-methine proton at 4.42 ppm and the methylene signal at 4.13 ppm (**7**, Fig. S15A). The SEC analysis revealed the expected decrease in the $M_{n,SEC}$ for **7** (4900 g/mol, Fig. S15B).

Based on these findings, it was proposed that sequential NCL – thiol-Michael addition could be employed to synthesise 3-arm mikto-arm star terpolymers, using a thioester functional polymer in the NCL step. To this end, an acid-functional PEtOx[37] (PEtOx-COOH, M_n = 3100 g/mol) was synthesised and transformed into its thioester (PEtOx-COSPh; **8**). Integration of the aromatic signals from the thiophenyl group at 7.38 ppm in the ^{1}H NMR against the methylene side-chain signal at 2.32 ppm revealed that 95% of the chains contained the thioester group (Fig. S16). This was supported by coinciding traces from the DRI and UV (λ = 250 nm) detectors during SEC (Fig. S17). Using a fresh batch of PEG-cysteine (**2**), NCL was conducted with PEtOx-COSPh (**8**). The formation of the NCL product **9**, formally a PEG-PEtOx block copolymer, was confirmed by disappearance of the aromatic proton signal in ^{1}H NMR, which coincided with quantitative downfield shift in the methine signal of the cysteine end group of **2** from 3.95 ppm to 4.53 as well as appearance of the ethylene glycol signals at 3.50 ppm (Fig. S18).

Table 1. Summary of polymer modifications and conjugations.

Code	Reaction	Polymer	DF [%]	$M_{n,}$[c] [g/mol]	$Đ_m$[c]
1	Amidation	PEG-FTzCA	> 99	3700	1.06
2 (+2')	Deprotection	PEG-cysteine	> 99	6800	1.13
3 (+3')	NCL	PEG-b-BocPhe	> 99	7100	1.18
3[a]	NCL	PEG-b-BocPhe	> 99	4400	1.12
4	TMA[b]	**3** + TFEA	91	6600	1.05
5	TMA[b]	**3** + oligo(MeOx-alt-AA)$_n$A	96	4700	1.07
6	TMA[b]	**3** + oligo(EtOx-alt-AA)$_n$A	> 99	4800	1.07
7	TMA[b]	**3** + PEGA	> 99	4900	1.09
8	Thio-esterification	PEtOx-COSPh	95	5400	1.10
9	NCL	PEG-b-PEtOx	>99	9600	1.31
10	TMA[b]	**9** + oligo(ButOx-alt-AA)$_n$A	94	11900	1.13

[a] DMPP reduced; [b] TMA = thio-Michael addition; [c] Determined by SEC; DF = degree of functionalisation.

Molecular weight analysis revealed a multi-modal distribution with the corresponding peaks associated with the oxidised and

reduced adducts **9** and **9'** ($M_{n,SEC}$ = 9600 g/mol; $Đ_m$ = 1.31, Fig. S19) as well as unreacted PEtOx, which was not removed during dialysis against water (nMWCO = 3500 g/mol) (Fig. 5A**,** black). The presence of the oxidised adduct **9'** was again confirmed by addition of DMPP to the mixture of **9** and **9'** which resulted in a decrease in the molecular weight and a significant change in peak shape due to reduction of **9'** to **9** ($M_{n,SEC}$ = 7200 g/mol; $Đ_m$ = 1.21, Fig. 5A, red). To complete the synthesis of the 3-arm mikto-arm star polymer, thiol-Michael addition of **9** to a distinct acrylic acid terminated NPAE macromonomer (oligo(ButOx-alt-AA)$_n$A, $M_{n,SEC}$ = 1100 g/mol, Fig. S20) was performed.[39] Successful thiol-Michael addition was confirmed by comparing the ^{1}H NMR spectra of the product **10** with that of the oligo(ButOx-alt-AA)$_n$A, which revealed incorporation of the proton signals arising from oligo(ButOx-alt-AA)$_n$A (Fig. 5B).

The extent of the thiol-Michael addition was found to be 94% which was quantified through comparison of the integrals arising from the methine proton at 4.42 ppm, from the terminal cysteine residue in the parent polymer **2**, with the signals at 1.46 ppm corresponding to a methylene environment in the side-chain of the oligo(ButOx-alt-AA)$_n$A. Initial SEC analysis of the crude product revealed a bimodal distribution (M_n = 9800 g/mol and $Đ_m$ = 1.32, Fig. S21) with a low molecular weight shoulder peak present again attributed to the excess PEtOx carried forward from the NCL reaction. Upon purification by centrifugal filtration (nMWCO = 10000 g/mol) a well-defined mikto-arm star terpolymer **10** (M_n = 11900 g/mol and $Đ_m$ = 1.13) (Fig. 5A, blue) was obtained.

Conclusions

In conclusion, sequential NCL and thiol-Michael addition has been demonstrated to be a viable route to synthesise functional (co)polymers. A terminal cysteine-functional polymer predisposed for NCL was readily synthesised from cysteine. Efficient sequential NCL and thiol-Michael addition was realised using a phenylalanine thioester (BocPheSPh) and trifluoroethyl acrylate (TFE) as model compounds. Quantitative NCL and efficient (> 90%) thiol-Michael addition was achieved as confirmed by ^{1}H NMR, SEC and MALDI-ToF-MS. The efficiency of the thiol-Michael addition reaction was retained when TFE was changed to polymeric acrylates. The resulting (co)polymers are interesting models for bioconjugation whereby the architecture (Y-shaped vs linear) and composition of peptide/protein-polymer conjugates can be controlled by the selection of the individual components. Finally, using a thioester functional polymer in the NCL reaction, this approach has been employed to synthesise functional terpolymers in the form of a 3-arm mikto-arm star terpolymer, alluding to the potential of this methodology towards the synthesis of functional (co)polymers.

Conflicts of interest

There are no conflicts to declare.

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